Germinating potential of *Phyllanthus amarus* seeds: evaluation of biochemical parameters

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Abstract

Collection, authentication, optimisation of storage conditions and selection of suitable propagules for propagation and conservation are crucial in the case of wild-growing medicinal plants which have adaptive variations under different climate regimes. The present study aims to understand the factors (here, seed types and storage conditions) that influence the germination potential of wild-collected *Phyllanthus amarus* L. (Euphorbiaceae) using biochemical characteristics of seeds, besides taxonomical authentication of the species. The amplicons of 1150 bp in SCAR marker analysis and 6–7 ribs on seed surface shown by scanning electron micrograph confirmed that the wild-collected specimen is *P. amarus*. Interestingly, the mature green seeds harvested and shade-dried within 0–3 days exhibited maximum sink percentage (55.8%) and showed a significantly ($p < 0.001$) higher germination percentage with carbohydrate and proteins than the float seeds. However, the float seeds contained 33% less fat content than the sink seeds. Upon storage, the germinability of *P. amarus* seeds was found to be 12 months at 4 °C without moisture. This shows that viable seeds can be harvested and stored appropriately to ensure longevity. This study makes a case for the preservation of seasonal, short lifespan seeds such as *P. amarus*.

Keywords *Phyllanthus amarus* seed · Germinability · Longevity · Storage

1 Introduction

The medicinal herb *Phyllanthus amarus* L. (Euphorbiaceae) is widely distributed in tropical and subtropical regions of Central and South Asia [1]. It is one of the most essential ethnomedicinal herbs that is orally administered for jaundice, asthma, hepatitis and malaria, and is known to have diuretic, antiviral and hypoglycaemic properties [2]. The whole plant extract is reported to contain alkaloids, flavonoids, phenols, coumarins,

tannins, terpenoids and lignans are responsible for its curative properties [3]. More than 60% of the people in the developing countries depend on the herbal drug for their ailments [4]. Despite having medicinal uses, this medicinal plant is not cultivated, so it has been gathered from wild sources and its availability will be challenged by herbivorous overgrazing [5] and climatic change [6]. Therefore, the species is likely to be extinct or loss of its habitat at a faster rate [7, 8], so its long-term conservation is an important criterion to be considered

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[9]. Recently, researchers recognised the value of conserving the medicinal plant, but they are facing several challenges that hampered their efforts [10]. As the wild-grown *P. amarus* has endured adaptive variation under different climate regimes, [11] germinability potential of seeds and its biochemical nutrients constituents under natural habitats remains a threatening reality for in situ conservation of the species.

Though the medicinal plant has been distinguished according to the morphological features by the taxonomist, the species *P. amarus* is often mistaken for other closely related species such as *Phyllanthus niruri*, *Phyllanthus debilis*, *Phyllanthus maderaspatensis* and *Phyllanthus virgatus* [12]. In spite of taxonomic keys available for identification of *P. amarus*, many of the *Phyllanthus* species are invariably collected under the name *P. niruri* because all the species share a common Tamil vernacular name 'Keezhanelli'. Thus, these species are used interchangeably by local collectors. [13]. However, a number of publications from India use the name *P. niruri*, which actually is endemic to America, only [12]. The *P. niruri* described in the Flora of British India [14] has been reported to be a mixture of three closely related but distinct species, namely *P. amarus*, *P. debilis* and *Phyllanthus fraternus*. Thus, it is not uncommon to find admixtures of related/allied species and sometimes also of other unrelated genera. The consequences of species admixtures can range from reduced bio-efficacy of the drug to lower the trade value as well as threats to the very safety of herbal medicines [15]. Earlier studies have attempted to resolve the nomenclatural problem persisting with *P. amarus* for easy identification of the related species by analysing the morphological, anatomical characters and sequence-characterised amplified regions (SCAR) markers [16].

Most annual medicinal plants are seasonal and their availability primarily relies on viability and successful germination of seeds. However, the extent of seed viability is influenced by various environmental factors such as temperature, relative humidity, salinity, light and soil moisture. [11]. Typically, the fresh seeds of *P. amarus* show less than 50% germination and the viability is found to decrease significantly with storage time [17]. As low-quality seeds can reduce the seedling percentage and seedling emergence rate [18], the seeds need to be harvested at the right stage of maturity. More importantly, the seeds should be dried appropriately for storage [19–21]. The foremost problem is improper seed storage conditions such as low room temperature and adequate moisture can promote fungal growth [22] and insect development in seeds [23], which shortens seed viability [24]. The standard method to overcome this problem is proper maintenance, which has been demanded the optimistic seed viability for a longer period that is very essential to conserve the species.

Seeds accumulate carbohydrates, proteins and lipids in different proportions depending on the species in a given condition. During seed development, the reserve material is utilised and weight is gained for them to sink in water. This phenomenon mainly depends on the assimilate level and amount of reserve material [25]. The inherent moisture level will be the lowest in mature seeds and highest in the initial stage. Initially, reserve materials are loaded heavily with carbohydrates and lipids (non-polar), and in later stages of development, the lipids become negligible [26]. Alpha-amylase and the accompanying hydrolytic enzyme action on the starch are the predominant form of glucose, which is then utilised for the growth of tissue, shoots and roots from the seeds [27].

Based on the above background, it has been observed that identifying and harvesting seeds at a suitable stage of development and drying and storing them affects the viability of the seeds, but there has been no study to address this problem in *P. amarus* so far. Therefore, this study has been conducted in order to find optimal seed storage conditions with reference to factors that limit the seed germinability of authenticated *P. amarus* by morphological and molecular basis.

2 Materials and methods

2.1 The study area, collection and identification of *P. amarus* seeds

Wild plants of *P. amarus* were collected from Nandhi Nagar, Dharmapuri District, India, which lies between 12° 6' 23.4972" N 78° 8' 10.1076" E at an altitude of 340 m above MSL. A random sampling was done only in the uncultivated wastelands since additional water and nutrients from cultivated fields adversely influence the nutritional composition of seeds. Each sampling included ~ 100 plants (per location) which were free from any pest/disease, herbivorous disturbance and human activities. Every sampling location included three sampling points in proximity, within ~ 100 m². None of the collection sites was inside the national park or restricted forest area, so prior permission was not required for the collection of *P. amarus*. The collected specimens were soon taken to the lab in a zip-lock cover for observation and processing. The mature seeds were harvested from the randomly selected shade-dried plants. It was observed that the number of seeds in the field-collected plants varies from ~ 50 to 150.

2.2 Storage of seeds

The collected seeds were stored for a specific duration of time and they were checked periodically for its germinability.

Initially, we separated the seeds that sink and float in water for its germination differences, with respect to imbibition time. The unsorted seeds were stored in four different storage conditions such as seeds with moisture in room temperature (set 1), without moisture (set 2) under room temperature, seeds with moisture at 4 °C (set 3) and seeds without moisture under 4 °C (set 4) under refrigeration. Moisture-free conditions were achieved by removing the excess moisture in the ambience by using self-indicating dry silica gel beads 100 g for desiccation [28] and for sustaining moisture, tissue paper soaked in distilled water in sealed plastic containers [29]. The water-soaked tissue paper was replaced every 2 weeks, and silica beads were replaced when required (based on colour change). After every month, the germination percentage was checked for 100 seeds in triplicate from each set.

2.3 Moisture content

To measure the moisture of the seed, the association of official chemists method was followed [30]. Briefly, fresh sample weight (seeds) was recorded and placed them in the hot air oven until it reached a constant weight. Moisture percentage was calculated as:

$$\text{Moisture (\%)} = \frac{\text{Fresh weight(g)} - \text{Dry weight(g)}}{\text{Dry weight(g)}} \times 100$$

2.4 Morphological and molecular identification of *P. amarus*

Initially, the wild-collected specimen was identified by its characteristic feature of blunt leaf tip (Fig. 1a). The prepared voucher specimens were sent to the Botanical Survey of India (BSI), Coimbatore, India, for authentication and were deposited in the herbarium at the Centre for Advanced Studies in Botany, University of Madras (No: CASBAH1014). The fresh sample (leaf) was taken for genomic DNA isolation [31] for molecular validation. In accordance with Jain et al. [16], forward (5'-AGAATTCGTATCTTCGTATACGTCATGA 3') and reverse (5'-AGAATTCGGTTCAAGCACAGCGGAAGAAG 3') primers were synthesised (M/s. Synergy Scientific Services, Bangalore, India) and used as a sequence-characterised amplified regions (SCAR) marker for species verification. The PCR reaction master (25 µL; M/s. Ampliqon, Odense, Denmark) contained 1 µL DNA template (170 ng), 12.5 µL PCR buffer, 2.5 µL both forward and reverse primers (10 pmol) and 6.5 µL sterile water. The PCR was carried out in a DNA Peltier thermocycler programmed for initial denaturation at 94 °C for 5 min and 40 cycles of 94 °C for 1 min, 38 °C for 1 min and 72 °C for 2 min, with a final extension of 72 °C for 5 min. The amplified products were separated on 1.2% agarose gel containing 0.5 µg/mL of ethidium bromide and the gel was photographed using a gel documentation system (Bio-Rad Laboratories Inc, USA). The whole seed was measured and documented for its dimensions and structure in

Fig. 1 Species of *Phyllanthus* in their natural habitat (top row) and shape of their leaves (bottom row). **a** *P. amarus*; **b** *P. debilis*; **c** *P. madraspatensis*

scanning electron microscopy (SEM; HITACHI, Model S3400, Japan). The mature sink seed was dehydrated with graded ethanol (10, 20, 40, 60, 80 and 100%, respectively) at an interval of 2 min and air-dried for 10 min. The stub-fixed and gold-coated (10 nm thickness) seeds were directly viewed under an acceleration potential of 10 kV.

2.5 Germination of *P. amarus*

For the germinability test, 1 g of visibly healthy seeds (i.e. free of microbial colonisation, decay and herbivore damage) were separated into sink and float seeds by a method proposed by Edson et al. [32] and assessed using a method described by Karthik et al. [11]. A seed was considered as germinated when there was an emergence of the radicle, and the observation of seed germination was recorded daily.

2.6 Biochemical contents of the *P. amarus* seeds

Biochemical contents of both sink and float seeds were measured from 100 mg of seeds. The total protein, carbohydrate and lipid content of the seeds were estimated by following the method of Bradford [33], Dubois et al. [34], and Folch et al. [35], respectively.

2.7 Statistical analysis

All the laboratory experiments were carried out in completely randomized block design (CRBD), and each independent experiment was repeated at least three times with three replications for confirmation of the results. The effects of seed storage conditions were analysed by analysis of variance (ANOVA), and treatment means were compared by Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$. The difference between the seed types (sinking and floating) was analysed by Chi square (χ^2) tests and the treatment means were compared by DMRT at $p \leq 0.05$. The germinability response of seed storage under different conditions and time was assessed by the best-fit (regression) equation based on the highest R^2 value. All the graphs were constructed using SigmaPlot® (Ver. 10.0 for Windows), and statistical analysis was performed using RStudio® (Ver. 1.1.456 for windows).

3 Results

3.1 Morphological and molecular identification of *P. amarus*

The morphology of the leaf tip is oval, which is a striking characteristic feature of *P. amarus* (Fig. 1) when compared

to related species. The appearance of a single band at 1150 bp using a species-specific SCAR marker on agarose gel confirming that the collected specimens were *P. amarus* (Fig. 2) according to Jain et al. [16]. Scanning electropherogram of *P. amarus* seeds shows that (Fig. 3) the shape of the seed is ovoid reniform (i.e. the length $[828 \pm 11 \mu\text{m}]$ slightly shorter than its height $[870 \pm 17 \mu\text{m}]$), comprising of 6–7 ribs that are regularly or irregularly arranged with longitudinal finger-like rodlets (hair). The rodlets measured $86.5 \pm 3 \mu\text{m}$ in length and a width of $10.4 \pm 0.3 \mu\text{m}$, which make a permanent imprint on the surface of the seed (Fig. 3c). The hilum arising from the tracheid bar (length of $123 \pm 4 \mu\text{m}$) is situated in subapical position on the seed (Fig. 3d).

3.2 Germination of *P. amarus* seeds

Seeds harvested from the shade-dried plants at 0–3, 4–7, 8–15 and 16–30 day intervals were subjected to buoyancy and germination tests. The result shows that the sinking rate ($y = 134.72e^{-0.795x}$; $R^2 = 0.98$) and germination rate ($y = 60.83e^{-0.729x}$; $R^2 = 0.99$) were exponentially decreased with respect to the duration of drying period with significance (Table 1). The maximum number of sinking seeds ($58.0 \pm 4.39\%$) and highest germination percentage ($29.0 \pm 3.10\%$) were noticed in the seeds that were harvested within 3 days of shade-dried plants (Table 1). To achieve the maximum seed germination, the optimal time of the imbibition was established by soaking the seeds (both sink and floating seeds separately)

Fig. 2 Molecular conformation of *P. amarus* using SCAR marker (**M**: Marker 100 bp ladder; **P1**: Amplified PCR product 1150 bp conforming *P. amarus*, **P2**: *P. debilis*; **P3**: *P. madraspatensis*)

Fig. 3 Morphology of *P. amarus* seeds by using scanning electron microscopy. **a** structure of the seed; **b** dimensions of the seed; **c** closer view of the finger-like rodlets; **d** closer view of embryo of the

seed. The top arrow mentioned in the figure is hilum and the bottom arrow indicates tracheid bar

Table 1 Time period of shadow drying for collection of viable seeds

Collection of seeds (days)	Sink (%)	Germination (%)
0–3	58 ± 4.39 ^a	29 ± 3.10 ^a
4–7	27 ± 3.63 ^b	13 ± 2.08 ^b
8–15	15 ± 2.23 ^c	6 ± 1.41 ^c
16–30	6 ± 1.41 ^d	2 ± 0.81 ^d

Germination here is the total seeds that include both sink and float seeds. Error bars in the line graph indicate the standard error. The alphabets showing different are the data that are significantly different from each other

harvested between 0 and 3 days at 2, 5, 10 and 24 h. The result of sinking seeds shows that germinability logarithmically ($y = 3.91 \ln(x) + 52.23$; $R^2 = 0.99$) over time from $52.3 \pm 1.45\%$ and it was maximum ($57.7 \pm 0.9\%$) seeds soaked for 24 h (Fig. 4a). In the case of floating seeds, maximum germinability ($4.3 \pm 0.9\%$) was observed in seeds imbibed for 5 h and the germination decreased later when prolonging the soaking time. The study did not find any significant variation in germination

percentage among the sink and float seeds of different imbibition periods, invariably germination was noticed 13-day after plating.

3.3 Storage of *P. amarus* seeds

To protract germinability, the sink seeds harvested between 0 and 3 days were stored in different temperature and moisture conditions, and the germination percentage was checked after every month. The result shows that there is a significant variation among the seeds stored when subjected to different temperature and moisture (Fig. 4b). Seeds stored at room temperature both RT + M (room temperature with moisture) and RT reduced their viability drastically and completely lost their germinability within 4 months. However, seeds stored at 4 °C showed extended viability than their counterparts. Germinability was comparable up to five months with 4 °C + M stored seeds, nevertheless the seeds stored at 4 °C edge over later and have 2 months extended germinability up to 12 months.

Fig. 4 Comparison of *P. amarus* seeds germination percentage of at different time of imbibition (a) between sink and float seeds and duration of storage conditions for the unsorted seeds (b). The storage conditions such as room temperature with moisture (RT+M), room temperature without moisture (RT), 4 °C with moisture (4 °C+M) and 4 °C without moisture (4 °C). Error bars in the line graph indicate the standard error. Horizontal line in the subplot boxes indicate mean (thick) and median (thin) germinability, lower boundary of the box indicates the standard deviation while the upper boundary of the box indicates 95% percentiles. The lower and upper whiskers represent the minimum and maximum observed value. The alphabets showing different are the data that are significantly different from each other

3.4 Biochemical contents of the seeds

The result shows that the total protein and total carbohydrate content are significantly higher in the sinking seeds than floating seeds. But the moisture content and the lipid content were significantly lower in sink seeds than in floating seeds (Fig. 5). Among the biochemical contents of the seeds, the maximum variation was observed in the carbohydrate levels of the sink seeds (62.78%) which is ~ 51.6%

Fig. 5 Biochemical constituents of sink and float harvested seeds of *P. amarus*. Error bars in the line graph indicate the standard error where $p < 0.001 = 99.99\%$ confidence, ns = not significance. The alphabets showing different are the data that are significantly different from each other

higher than in the floating seeds (41.43). Similarly, the total protein of the sink seeds (11.7%) was found to be three times higher than in the float seeds (2.8%).

4 Discussion

In trade, the medicinal herb *Phyllanthus* has a wide demand, but as a species, it is often wrongly identified and hence leads to unproductive benefit. Many of the *Phyllanthus* species belonging to the subsection 'Swartzian' grow in close proximity in India and their morphological identification is not easy. The confusion connected to the name *P. niruri* is due to Linnaeus's inclusion of synonyms which actually belong to different species, namely *P. amarus* Schum. & Thonn., *P. debilis* Klein ex Wild., *Phyllanthus madraspatensis* L., *P. urinaria*. The leaf shape character of *P. amarus* is oblong with rounded tip, whereas in *P. fraternus*, the leaf tip is obtuse. The *P. debilis* leaf is narrow elliptical in the upper region and cuneate at the lower end and the leaf tip is acute. The *P. maderaspatensis* leaf is spatulate and its leaf tip is oblong-elliptic [36]. The *P. urinaria* leaf is oblong, mucronate below leaves reddish-green seeds is seen [37].

To distinguish the *Phyllanthus* species, Jain et al. [16] developed SCAR markers for molecular characterisation of four species of the niruri complex (*P. amarus*, *P. fraternus*, *P. debilis* and *P. urinaria*) using MAP 09 and MAP10 primers. The MAP primers exhibited a distinct polymorphism that

are species-specific DNA fragments, 1150 bp for *P. amarus*, 317 bp for *P. fraternus*, 980 bp for *P. debilis* and 550 bp for *P. urinaria*. The results obtained by them were in accordance with the result of the present study, with 1150 bp amplicon obtained with field-collected *P. amarus* (Fig. 2).

Based on the SEM seed topology study of Machado et al. [38], only *P. amarus* seed consists of six-to-nine regular longitudinal ribs with transversal finger-shaped rodlets than its counterparts *Phyllanthus tenellus* Roxb., *P. niruri* L., *Phyllanthus stipulatus* (Raf.) Webster, *P. urinaria* L., *Phyllanthus carolinensis* Walt. The present study using SEM also resulted in the distinguishable presence of six-to-seven regular longitudinal ribs and confirmed the collected seeds as *P. amarus* (0.8 mm long and 0.8–0.6 mm width) whereas *P. debilis* (Seeds 1 mm long and 0.6 mm wide, longitudinally 6–7 ribs and coloured pale yellowish-brown) [39] and *P. madraspatensis* are trigonous with 1.5 mm length and a shiny dark-brown colour [40].

The germinability of natural *P. amarus* seeds was low. Therefore, harvesting and storage at the right time is very essential to produce good germinable seeds. The present study demonstrated that the seeds harvested within 0–3 days were matured and separated them from the plants. These also had a higher sink percentage and good germinability. Based on the conceptual study of Unander et al. [17], the germination percentage and duration of shade drying of *P. amarus* plants could be related to the dehiscence colour of the seeds. All the seeds collected within the first 30 h were dark green in colour and had more sink seeds and better germination (Table 1). Seeds collected after 96 h are light tan in colour and have significantly fewer sink seeds and lower germination percentage. From the results, it was apparent that the collection of dark green sink seeds is necessary for successful germination. On the other hand, light tan float seeds result in poor germination and are to be avoided in ex situ conservation.

The results of seed storage conditions revealed that a warm and wet environment seriously affects the germinability of the *P. amarus* seeds. However, seeds stored at 4 °C can improve the viability (Fig. 4). Unander et al. [17] reported that *P. amarus* seeds have a longer viability of up to 18 months when stored at –20 °C but the germination percentage declines over time. The results of the present study also report a similar trend: seeds stored at a lower temperature have maximum viability with a decreasing seed germination rate. Earlier studies have found that the germination percentage of older seeds increase because a slightly longer period of storage could eliminate germination inhibitors; therefore, they tend to germinate faster when compared to fresh seeds [17]. Fresh seeds had seedlings within 13 days and then upon storage for 6 months at room temperature, the time required for the seedlings was drastically reduced to only 10 days. Although, the rate

at which the germination takes place was quick and their germination success was dropped in all conditions.

From the earlier report of Pius et al. [41], the biochemical composition of *P. amarus* seeds very similar our sink seed composition (Fig. 5). The starch stored inside the seeds remains insoluble in water [42], where the prime enzyme cell wall-bound invertase breaks it down into glucose during germination and helps the mitotic cell division [43]. Hence, the distribution and concentration of glucose are the main criteria which directly correlate to the maturity and gain of weight to the seeds that make them sink.

Due to the assimilation of stored lipids at the time of maturity, the concentration was low in the sink seeds which displayed a quick saturate in the water as they sink, whereas the float seeds remain immature and their initial stored lipids remain non-polar, thus helping them float. Moreover, the non-polar lipids prevent water into protoplasm and thus prevent germination. Our results in *P. amarus* sink seeds have less lipid content by 33% than that of the float. So, there might be difficult for the water to reach germplasm of the float seeds. Thereby, the germination of float seeds could not be accomplished [44]. As a result of germination, it was understood that mature sink seeds break down their stored lipids for complete development. The lipids are highly non-polar hydrocarbons which are insoluble in water as compared to polar substances like glucose, which solubilise with a saturation point of 1.1 mg/g of water. Due to the presence of higher lipid, it makes them float; further, it blocks the entry of water into the germplasm through the testa [44]. A significantly higher percentage of germination can be related to the presence of a higher total protein content in the sink seeds (Fig. 5) due to the presence of many active enzymes compared to float seeds which has lower level of protein in this species [11].

5 Conclusion

Habitat destruction of the medicinal plant *P. amarus* has encountered a serious threat due to urbanisation, climatic changes and their raw demand. As described in this study, *P. amarus* has a low germination rate and its germination rate drastically reduces with time. In order to conserve the seeds, both the selection of viable seeds and condition of storage are major factors in the longevity of the seeds. It was observed that sink seeds have the ability to germinate but not float seeds. Sink seeds ratio was high during the first 3 days of shade drying. Storage at 4 °C without moisture resulted in seed viability for up to 12 months. So, this information provides a suitable parameter and criteria for *P. amarus* germination.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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